STUDYING REGUATION OF NEU5AC (SIALIC ACID) BIOSYNTHESIS IN DROSOPHILA

A Senior Scholars Thesis

by

DHEERAJ PANDEY

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2009

Major: Biochemistry and Genetics

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Approved by:

Research Advisor: Vlad Panin
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Studying Regulation of Neu5Ac (Sialic acid) Biosynthesis in Drosophila. (April 2009)

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Glycoconjugates are very important compounds found in all domains of life. They are not primary products of genetic code like other structural biomolecules such as proteins and nucleic acids. They are product of glycosylation, i.e. addition of sugars to target molecules like proteins and lipids. Sialylated glycoconjugates are synthesized when sialic acids from donor molecules are transferred to the target molecules by sialyltransferase. These sialylated glycoconjugates are involved primarily in mediating the cell-cell interaction. Due to their prominent position on cell surface they act as a recognition site for various receptors as well as pathogens. Until now, most of the research about sialic acids and their functions is limited to deuterostomes. The information about the biosynthesis of sialic acid is incomplete and not well studied in protostomes. In Drosophila, the GNE enzyme that catalyzes the first two steps of sialic acid synthesis in vertebrates, is absent but the sialylated products can be detected in small quantities. I studied if an isozyme of GNE is present in Drosophila which catalyzes the rate limiting reaction of the glycosylation pathway.

The results from immunoassay and western blot suggest that recombinant protein is being synthesized in the cytosol. At this moment, I am balancing the transgenic flies which will be tested for in vivo expression of GNE enzyme. I will perform physical tests to check for ectopic expression in these transgenic flies.

DEDICATION

Dedicated to my beloved Dad, Mr. Madan Raj Pandey, and Mom, Laxmi Pandey.

ACKNOWLEDGMENTS

I would like to extend my gratitude to all the individuals who inspired and helped me to carry out this research. I want to thank the Department of Biochemistry and Biophysics for giving me an opportunity to do the necessary research work and to use the departmental data and equipments. I am deeply indebted to my supervisor, Dr. Vlad Panin, from Texas A&M University whose help, stimulating suggestions and encouragement helped me during the time of research for and writing of this thesis. I am grateful to have a mentor like Dr. Naosuke Nakamura who instructed and helped me in every step of my research. I want to acknowledge my lab colleagues Elena, Dmitry, Apoorva, Caroline, Michiko and Dr. Kate Koles for their immense support. Especially, I would like to give my special thanks to my dear friends Neeraj K Aryal and Manoj Rajaure for their encouragement and support that enabled me to complete this work.

NOMENCLATURE

Neu5Ac	N-acetylneuraminic Acid
Neu5Gc	N-glycolylneuraminic Acid
GNE	UDP-GlcNAc 2-epimerase/ManNac Kinase
UDP	Uridine Diphosphate
GlcNAc	N-acetyglucosamine
N	Amino Group
C	Carboxyl Group
CTP	Cytidine 5’-triphosphate
CMP	Cytidine 5’-monophosphate
PEP	Phosphoenol Pyruvate

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CHAPTER I

INTRODUCTION

Glycosylation is an important biochemical reaction that requires enzyme to add sugars to proteins and lipids. The end products of this enzymatic reaction are called glycoconjugates. This process contributes to the diversification of biomolecules creating a “third language of life" following nucleic acids and proteins (1). Glycosylation is a part of set of possible post translational modification in some proteins that is required for their proper folding.

Sialylation is a type of Glycosylation in which sialic acid is added to the target molecule with the help of enzyme sialyltransferase. Sialic acids are a family of more than 50 structurally distinct molecules of 9-carbon–alpha-keto acids (2). Unlike most other sugar residue of vertebrate-glycocojugates which have five or six-carbon sugar backbone, sialic acids are composed of nine carbons (Fig. 1). They are instrumental to the great diversity of complex carbohydrates, which are frequently found on the surface of secreted glycoproteins and glycolipids (3). Sialic acids are located at non-reducing end of glycans attached to glycoconjugates, and appear

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This thesis follows the style of *Journal of Biological Chemistry*.

largely on cell surface in mammals and other vertebrates (Fig. 2). As a result of their location and negative charge, Sialic acids play an important role in mediating cellular recognition and cell adhesion. They are involved in the binding and transport of positively charged molecules as well as in attraction and repulsion of cells and molecules (4). There is evidence that sialic acids are prominently involved in cell-signaling mechanism (1). Other important functions include regulation and turnover of glycoprotein, pathogen-host recognition, immune system functioning, and nervous system development.

Figure 1.
Sialic acids are composed of 9-carbon–alpha-keto acids. Unlike most other sugar residue of vertebrate glycoconjugates which have five or six-carbon sugar backbone, sialic acids are composed of nine carbons.

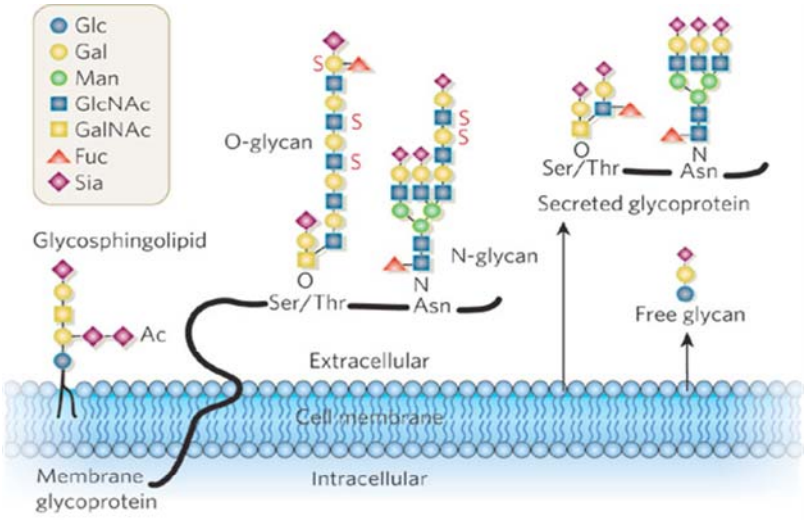

Figure 2.
Sialic acids are located at non-reducing end of glycans attached to glycoconjugates, and appear largely on cell surface in mammals and other vertebrates

The biosynthesis of sialylated glycoconjugates includes a number of enzymatic reactions that converts the precursor sugar into a donor molecule which is then used by a sialyltransferase for the linkage-specific sialylation of glycoconjugates. In vertebrates, UDP-GlcNAc is first epimerized to ManNAc which is then phosphorylated to yield ManNAc-6-phosphate by a bifunctional enzyme, UDP-GlcNAc 2-epimerase/ManNAc kinase. ManNAc-6-P is then condensed with PEP to give Neu5Ac-9-P catalyzed by Neu5Ac-9-P synthetase. Neu5Ac-9-P phosphatase removes the phosphate to yield Neu5Ac. Neu5Ac is then converted to active CMP-Neu5Ac using Cytidine 5'-triphosphate (CTP) by CMP-Neu5Ac synthetase. Finally, Sialtransferase (SialT) facilitates the addition of Neu5Ac to acceptor molecules (Fig. 3)

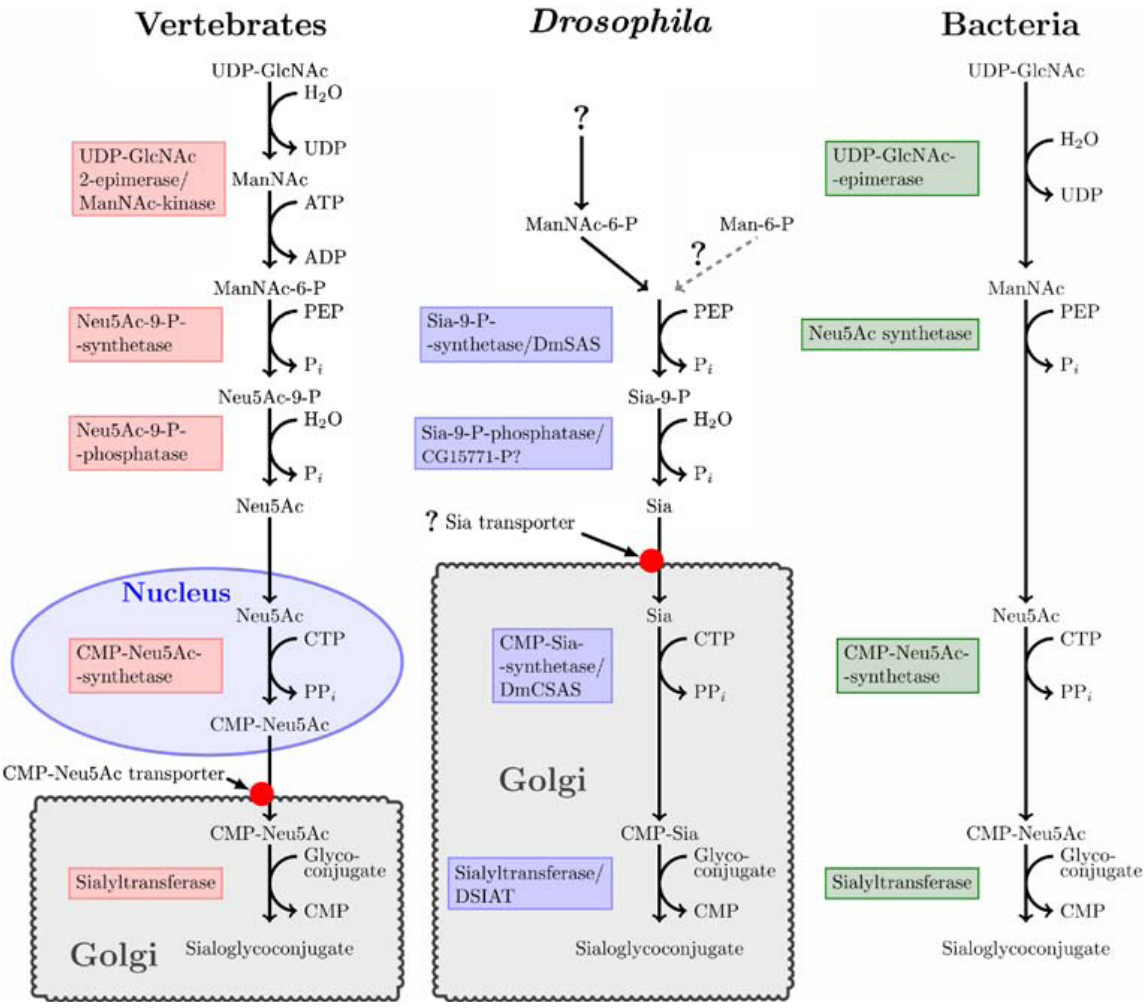

Figure 3: Sialylation pathway. *D. melanogaster* carries most of the enzymes necessary for sialylation except the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNac kinase that is necessary for the conversion of UDP-GlcNAc to ManNac-6-phosphate (precursor for Neu5Ac). Adapted from Glycoconj J DOI 10.1007/s10719-008-9154-4.

Sialic acid is found mostly in deuterostomes and some bacteria with exception of some protostomes. Deuterostomes mainly consists of vertebrates with some higher invertebrates like echinoderms. All other invertebrates including molluscs, annelids, and insects are protostomes. It may be possible that sialic acids are present in very limited quantities and restricted to particular tissues and stages in protostomes and other species

that appear to be lacking it. Studies have revealed that the genome of fruit fly *Drosophila melanogaster* lacks some essential genes of enzymes necessary for the synthesis of sialic acid (Neu5Ac), but contains several other orthologous genes believed to be involved in the downstream reactions in the biochemical pathway (2). Fruit fly is used as a model because it has a well studied genetic background for over 100 years. In addition, it has only a single copy of sialyltransferase gene compared with the mammals which have more than 20 different sialyltransferase. That makes it easy to study and genetically manipulate the biosynthesis pathway of sialylation in *D. melanogaster*. We know that *D. melanogaster* carries most of the enzymes necessary for sialylation except the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNac kinase that is necessary for the conversion of UDP-GlcNAc to ManNac-6-phosphate (precursor for Neu5Ac). Since the Fruit flies have most of the enzymes including SialT, and the sialylated product has been detected using mass spectrometry in very low amounts; it raises the question for origination of donor molecule. We are not sure whether *D. melanogaster* possess another non-homologous enzyme for that step, or it uses different precursor to bypass these first two steps of sialic acid synthesis. But this step looks like a bottleneck step in *D. melanogaster* sialic acid synthesis, which leads to the scarcity of sialylation. GNE is bifunctional enzyme, same as UDP-GlcNAc 2-epimerase/ManNac kinase, with characteristics of a fusion protein having N-term epimerase and C-term ManNackinase. The mutation in codons 263 to 266 of GNE leads to a disease called Sialuria or French type sialuria. It is an autosomal dominant inborn error of metabolism in which the feedback control mechanism in the biosynthesis of CMP-Neu5Ac is lost. To date, 7

patients have been reported with sialuria; presenting clinical features included mild psychomotor delay, coarse face, recurrent upper respiratory tract infections, and hepatomegaly (5).

Most of the information about sialylation is obtained from deuterostomes, and the evidence of sialylation in protostomes has been scarce. I am interested in finding out whether or not the sialylation is evolutionarily conserved. I am also interested in finding out its ancient function. For this project, I introduce a mammalian gene from rat that makes GNE enzyme to *D. melanogaster* to see if this enzyme can up-regulate the sialylation of target molecules, so that it would be easier for us to identify the target peptide for sialylation.

CHAPTER II

METHODS

Various biochemical and genetical assessment was employed throughout the length of my research. For the first part of my research, recombinant DNA technology was applied to insert the gene responsible for GNE enzyme into a vector. After the recombinant plasmid was prepared, it was introduced into Drosophila germ line and its cell culture. Second part of research will include biochemical assays to check the expression of the target molecules in the cell culture, and look at GNE expression phenotypes in the transgenic flies.

Recombination plasmid preparation

PCR (Polymerase Chain Reaction) was performed to amplify the genes for GNE wild type and GNE mutant with primers 5'-CCA CTA GTG TCG ACT GGT ACC GAT ATC-3' and 5'-CGA CGG CCG AGA AGA ACG GGA ATA ACC-3'. Gel electrophoresis was performed to check for the PCR products and eluted the appropriate DNA fragment of 2.4 kilobases. A digestion was set up for the purified PCR products (GNE-wt and GNE-mu) with restriction enzymes Eag I and Bgl II and cloning vector p3xFLAG-CMV (Fig: 4)with restriction enzymes Not I and Bgl II. A ligation reaction for plasmid vector and insert with T4 ligase was carried out. The vector with insert was then introduced into the host cells (*Escherichia Coli*) using electroporation. The transformed cells were then plated on a Petri dish for selection with ampicillin. Colonies

from the transformation was picked up and screened for GNE-wt-FLAG and GNE-mu-FLAG using PCR. The colonies with right insertions were selected and a mini preparation was set up to extract the purified plasmid with gene insert of my interest. GNE-wt–p3xFLAG and GNE-mu-p3xFLAG were amplified from CMV vector using PCR with primers 5'-GCC TCG AGC ATG GAC TAC AAA GAC CAT GAC GGT G-3'and 5'-CCA CTA GTG TCG ACT GGT ACC GAT ATC-3'. These inserts with 3xFLAG tag were re-cloned it into pMK-33 (an insect vector) and pUAST (fig 5) expression vectors. pMK-33 vector was digested with restriction enzymes Xho I and SpeI. Likewise pUAST vector was digested with restriction enzymes Xho I and XbaI. Both inserts with wild type and mutant variant gene were digested with Xho I and SpeI restriction enzymes. Vectors and inserts were then ligated. The constructs were further amplified by transformation and DNA purification. The prepared constructs were sequenced to check for the inaccuracy or any other mutations that may have resulted during cloning process.

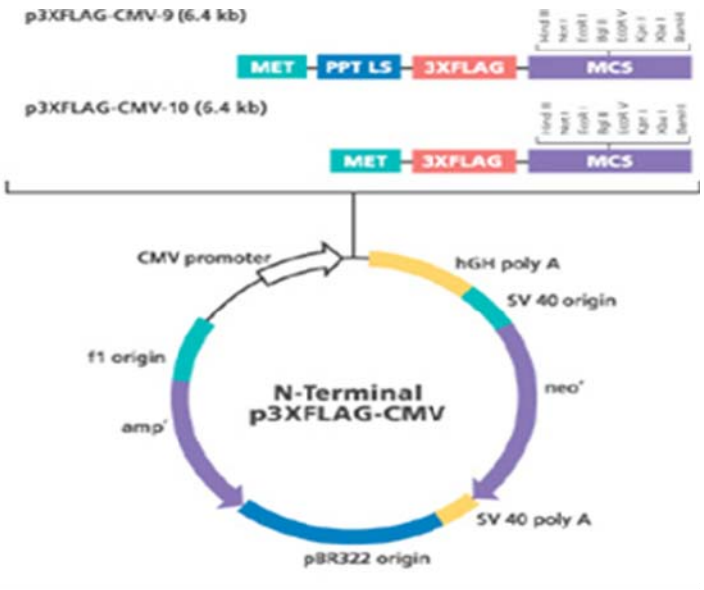

Figure 4: p3xFLAG-cmv cloning vector.

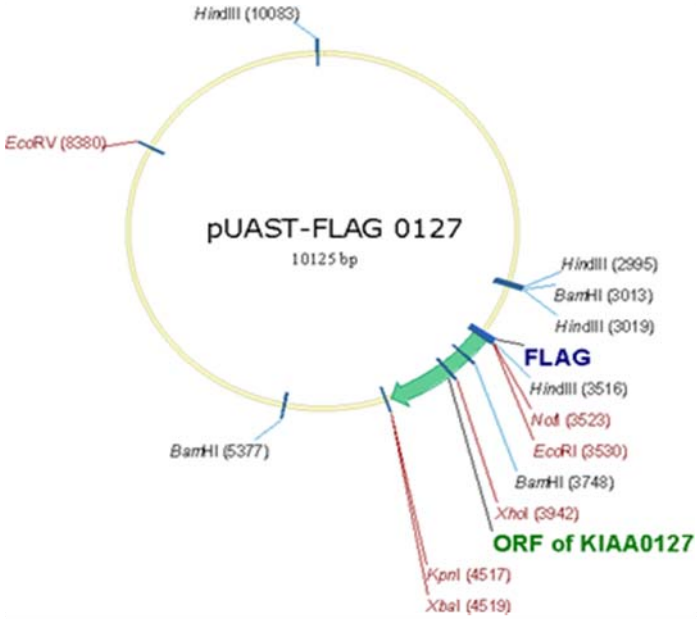

Figure 5: pUAST expression system.

Production of stable cell line

S2 *D. melanogaster* cells were transfected with the pMK vector with GNE-wt-p3xFLAG. The transgenic cells were selected using hygromycin. A transient and an stable line of cell culture was maintained. An immunoassay of transient line was performed to check for percentage of transformed cells. After several batches of selection and passaging, the stable line cells were analyzed for the recombinant protein with Western Blot. Various biochemical assays will be performed to check the expression of GNE in the recombinant cell cultures. These assays will include, but not be limited to α-FLAG immunostaining, and SNA lectin staining.

Production of stable transgenic germ line

The pUAST vector with GNE-p3xFLAG was inserted into *D. Melanogaster* germ line using P element insertion. Several transgenic lines with probable insertions in different sites were received. These lines were crossed with balancer flies with the genotype w^-; Bl/Cy; T_2/T_6 to find the physical location of inserts in chromosomes. Two different lines with insertion on each of X, 2^{nd} and 3^{rd} chromosomes were selected (total 6 lines). These flies will take several generations to be a stable line. Physical and behavioral tests on these flies will be performed once balancing process is over. In vivo expression of GNE enzyme and its products will be checked using different biochemical assays and mass spectrometry.

CHAPTER III

RESULTS

Recombination plasmid preparation

Gel electrophoresis was performed to check for the PCR products and eluted the appropriate DNA fragment of 2.4 kilobases for GNE-wt and GNE-mu (figure 6). DNA of interest with vector was extracted from transformed bacterial colonies with vector plasmid and insert by gel extraction (Figure 7). GNE with 3xFLAG tag was amplified using PCR (Figure 8). The results from digestion with several restriction enzymes gave the expected bands with right sizes (Figure 9). It means my insertions were of right size and in right orientation. The sequencing results which are not included in these results further proved my insertions do not carry unexpected mutations.

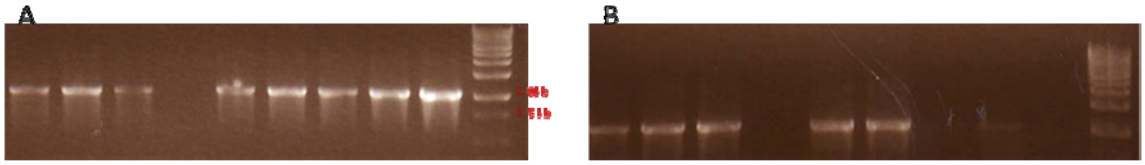

Figure 6: A) PCR of GNE wt colonies. B) PCR of GNE mu colonies. 1 kb ladder used.

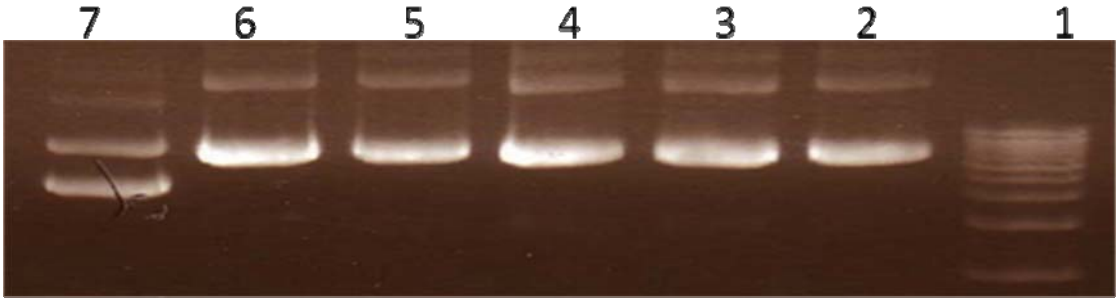

Fig 7: GNE-3xflag-CMV9 (6.4kb) minipreperation. Lane 1: 1 kb ladder; Lanes 2-4: GNEwt; Lanes 4-6 GNEmu. 1 kb ladder used.

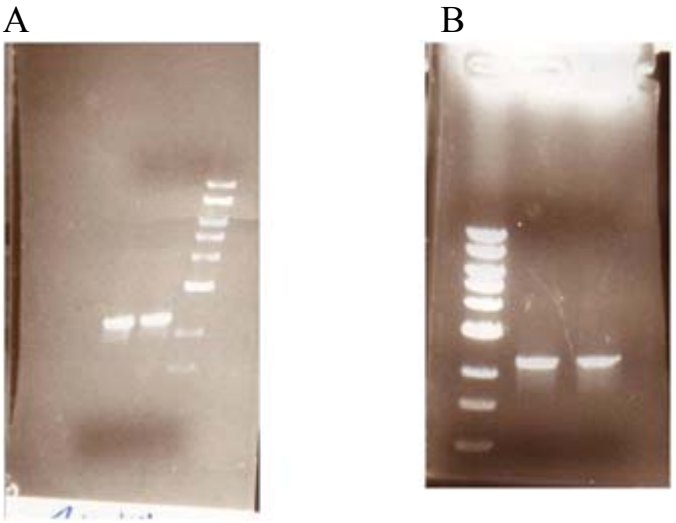

Figure 8: PCR of GNE with tag. A) GNEwt; B) GNEmu

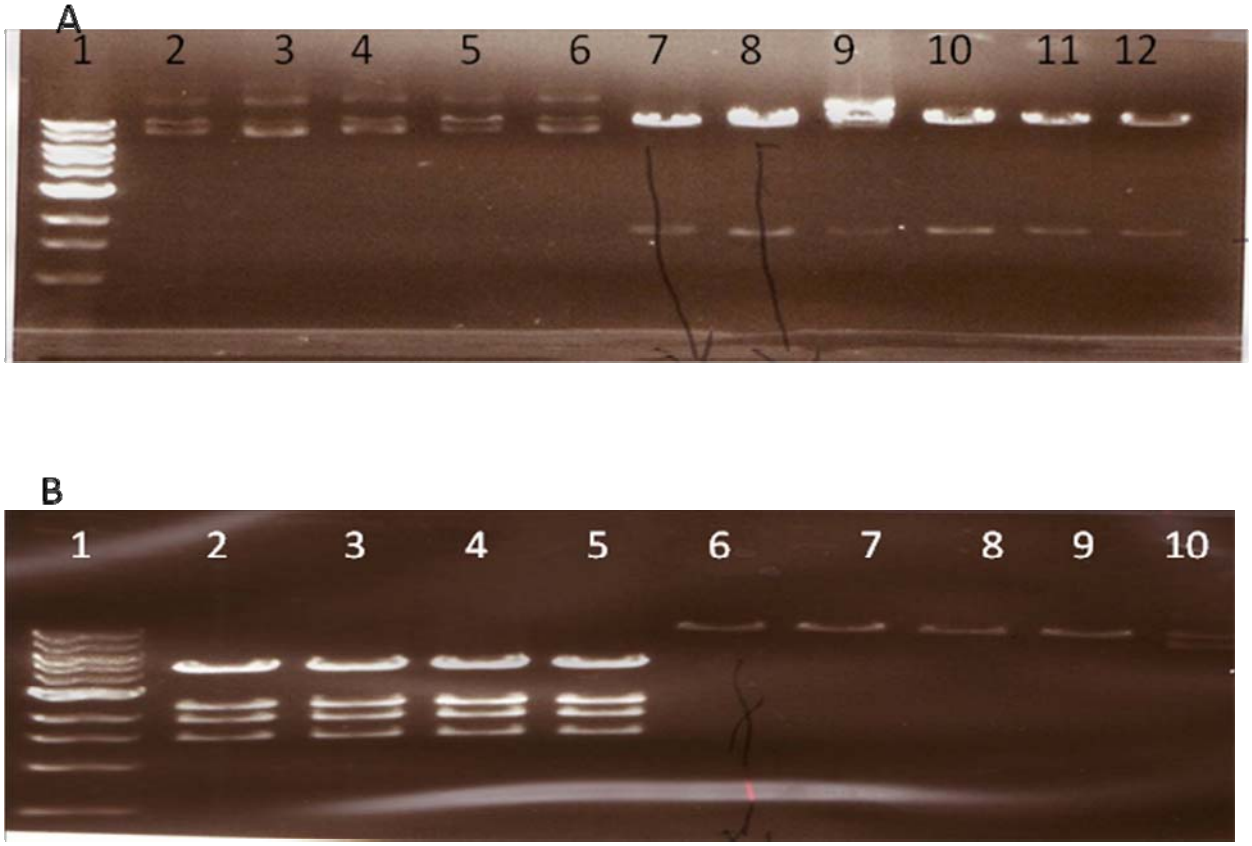

Figure 9: Digestion to check the vectors and inserts are cloned as desired. A) Lanes 2-6: pUAST- inserts digested with kpn1; Lanes 7-2: pUAST-inserts digested with EcoR1. B) Lanes 2-5: pMK- inserts digested with xho1 and EcoR1; Lanes 6-10: pMK-inserts digested with EcoRV. 1 kb ladder used.

Immunoassay and Western blot

The results from immunoassay and western blot suggest that recombinant protein is being synthesized in the cytosol (figures 10 and 11). Figure 11 illustrates that GNE enzyme is approximately 88 kD (2400 bases/3 *110 Da.). The recombinant protein is made even without inducing it with $CuSO_4$.

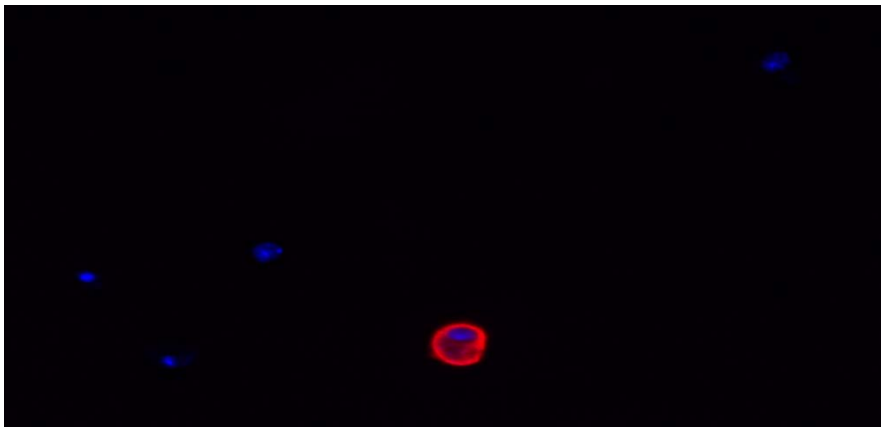

Figure 10: Transfected S2 cells illuminated with DAPI and Rhodamine simultaneously. Red coloration on cytosol is because of the presence of recombinant protein.

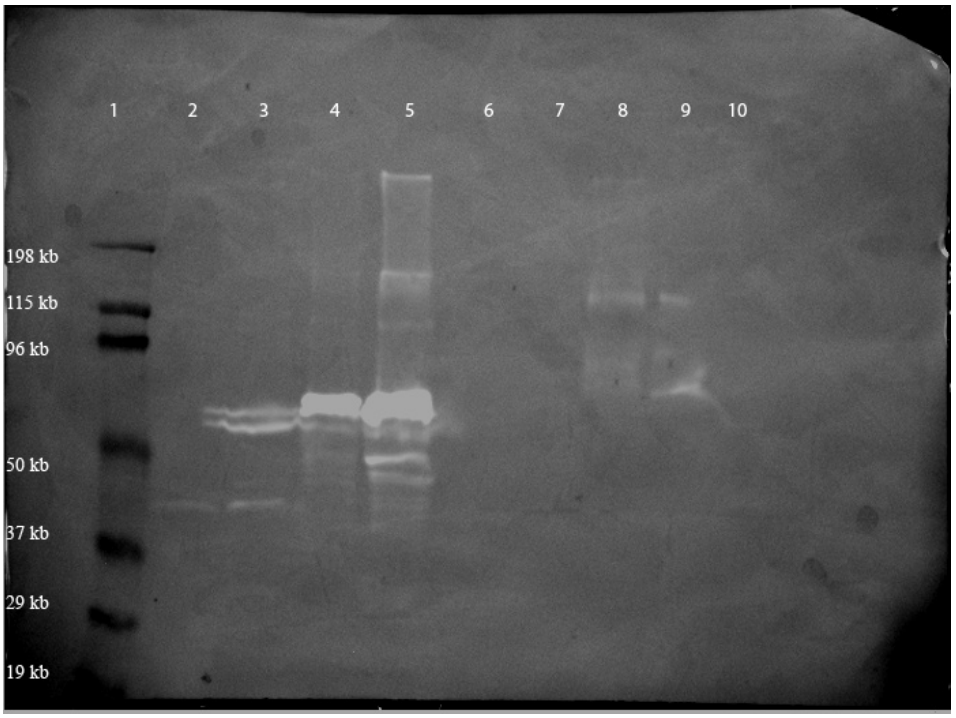

Figure 11: Western blot results for GNE recombinant protein. Lane 1 ladder (Pre-stained SDS- PAGE STDS); lane 2 S2 pellet, lane 3 S2 induced pellet, lane 4 GNE transfected S2 pellet, lane 5 GNE transfected S2 induced pellet, lane 6 S2 supernatant, lane 7 S2 supernatant induced, lane 8 GNE transfected S2 sup, lane 9 GNE transfected S2 sup.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Results from immunoassay and western blot suggest the GNE enzyme is being produced in cell culture. I have yet to perform other biochemical assays to determine if this enzyme will indeed up regulate the biochemical synthesis of sialic acid. Once the chromosome balancing is over, the expression of GNE enzyme in-vivo will be checked by dissecting the *Drosophila* larval brain. Next part of assay will check for the synthesis of ManNac-6-phosphate, which is the product for reaction catalyzed by GNE enzyme. This result will conclude that the donor for sialic acid synthesis in drosophila is same as in mammals. It would also mean that they have lost this enzyme in evolutionary process. On the other hand, if there is no production of ManNac-6 phosphate in transgenic flies, it could mean Drosophila by passes first two steps or it has a different donor molecule involved in sialic acid synthesis. If the results support my hypothesis, I will introduce mutant form of GNE enzyme in which negative feedback has been eliminated. It will affect the quantity of sialic acid synthesis because the product will not inhibit enzyme from catalyzing first two steps of synthesis, thus increasing the sialylated products.

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